Maturity of the Lamb Immune System

Honors Thesis

Presented to the College of Agriculture and Life Sciences,
Department of Animal Sciences
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By
Mabel Elizabeth Gailor
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Dr.Jerrie Gavalchin
Dr. Michael Thonney

ABSTRACT

Vaccines targeting specific threats to lamb health could be immensely instrumental in curbing high levels of lamb mortality in the U.S. lamb industry. However, the age at which vaccine administration would be most beneficial is currently unspecified due to limited knowledge of when lambs become immunocompetent. This study's main objective was to determine the effect of age on the immunological competency of sheep in order to determine the optimal schedule for vaccinating lambs. An experiment was designed to examine the ability of lambs of varying ages to mount an antigen-specific immune response against Keyhole Limpet Hemocyanin (KLH) after vaccination with KLH in 10% aluminum hydroxide as the adjuvant. Groups of five lambs were vaccinated subcutaneously with one of three treatments (vaccine, control, adjuvant only) three times at two week intervals over a total of six weeks. The age at administration of the treatment was also variable (n=5); the lambs were either 0, 5, or 40 weeks of age at the start of their respective six week vaccine trial. Three blood samples were taken immediately prior to the second and third treatment and two weeks after the third. Lymphocyte proliferation, and production of total immunoglobulin (Ig), and KLH-specific Ig were measured. An increase in lymphocyte production in vaccinated animals in response to vaccines was seen as early as 4 weeks of age, after two vaccines. The production of anti-KLH Ab in vaccinated lambs began after the first vaccination and had a fold response four times as high as control and adjuvant-only animals when sampled two weeks after the first vaccination.

ACKNOWLEDGEMENTS

I'd like to thank my thesis and academic advisor, Dr. Jerrie Gavalchin for guiding me through not only my thesis but my undergraduate career at Cornell, for patiently fielding all of my questions, and for allowing me the opportunity to experience research as an undergraduate.

I would also like to thank Dr. Michael Thonney for helping me come up with this thesis topic, and for assisting me with the sampling and analysis of the data. It was a great experience to work on his "simple, yet elegant" project. I would also like to thank Bryan McGee and John Knowlton at the Cornell TNR center for enduring the cold throughout December and January to collect blood samples with me.

Finally, I'd like to thank my friends and my family for loving and supporting me throughout this academic endeavor.

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LIST OF ABBREVIATIONS USED

Ab: Antibody

KLH: Keyhole Limpet Hemocyanin

ConA: Concanavalin A

PWM: Pokeweed Mitogen

Ig: Immunoglobulin

Introduction

Lamb health is a major area of concern in the lamb meat industry as lamb mortality can be a limiting factor in the profitability of sheep operations. Mortality rates are estimated to be between 15 and 51% with rates as high as 35% considered acceptable among large sheep operations (Daniels et al., 2000). When experiencing acute disease outbreaks, losses approaching 100% have been observed (Shelton and Willingham, 2002). To help avoid and prevent such economically detrimental events, it is critical to understand the development of the lamb's immune system in the first few weeks of life in order to determine the appropriate age at which vaccines can be administered to generate protective immune responses.

Current immunization practices focus on immunizing the pregnant ewe before parturition to confer the best possible repertoire of antibodies (Ab) and passive immunity to the lamb via colostrum (reviewed in Butler, 1999). Colostrum contains antibodies to diseases prevalent to in a specific flock, and to diseases that an ewe has been vaccinated against, and helps prevent disease outbreak in lambs. Although this passive immunity works well to protect most lambs against common infectious agents for the first ten to twelve weeks of life, it is dependent both on the strength of the ewe's immune system and the quantity of colostrum received by the lamb. Thus, there can be a high degree of variability in the quality of passive immunity acquired by lambs. Determination when immune competence develops during the crucial first weeks of life will identify the earliest time point for effective vaccination to help prevent some of the infectious diseases responsible for postnatal lamb losses. Further, the ability to launch an antigen-specific active immune response would provide the lamb with the opportunity to protect

itself against virtually any possible antigenic threat, independent of passive immunity from the ewe..

The objective of this study was to examine the age at which immunological competency is achieved in postnatal Finnsheep x Dorset lambs. To investigate this, lambs were vaccinated with KLH in alum or alum alone at 0, 5 weeks, or 40 weeks of age and the KLH-specific cellular and humoral responses measured in terms of leukocyte proliferation, total IgG, and KLH specific IgG production.

Review of the Literature

Much of the previous research on the immune system of the lamb has focused on the importance of passive immunity from the ewe in protection from infections. Passive immunity is conferred from ewe to lamb through colostrum in the first 24h after birth. The level of immunity provided by colostrum is related to the level of systemic immunity of the ewe (G. Chappuis et al., 1998). Thus, previous research in lamb immunity has focused on strategies to provide effective passive immunity towards commonly encountered pathogens in sheep flocks. A definitive study examining the immunocompetency of lambs in response to vaccination at varying ages is needed to determine when the immune system of the lamb is able to produce a protective immune response including antibodies.

Vaccination studies in the 1960's testing intramuscular immunization of newborn lambs found that the vaccines were ineffective, presumably due to the blocking effects of maternally-derived passive immunity (Mutwiri et al., 2000). In a later study, De la Rosa et al (1997) found that vaccinating pregnant ewes imparted protective immunity in lambs against enterotoxemia (a common pathogen) for 12 weeks, and the vaccination of the lambs themselves provided no added immunological protection. Again, it was concluded that vaccine-induced antibody production in the neonatal lambs was suppressed by the presence of maternally-derived antibodies, as the immune response in vaccinated lambs was diminished response whether or not their dams had been vaccinated.

In contrast to De la Rosa's work, Fahey and Morris in 1978 found that fetal lambs were, in fact, capable of launching antibody responses of varying characteristics in terms

of Ig type and magnitude *in utero,* with the amount of antibody produced and persistence of the response increasing as the lamb aged. Also in support, several studies have shown that early in life ruminants are capable of responding to antigenic threats (Mutwiri et al., 2000); suggesting that they should be capable of generating immune responses to antigens.

There is a period of time when neonates contain too much maternal antibody in their system, preventing them from responding to a vaccine but not sufficient to protect against states of disease. Ideally, vaccines should be administered to an animal at the age at which it first becomes susceptible to infection, prior to the decline of passive immunity (G.Chappuis et al., 1998). Previous studies suggest that a major obstacle to successful vaccination of young lambs is the presence of blocking levels of maternally- derived antibodies. This blocking effect of maternal immunoglobulin may be overcome if the antigenicity of a vaccine is improved over that of traditional vaccines, or when a naïve (to the lamb's system) vector is used to present the antigen (G.Chappuis et al., 1998).

Strategies using multiple boosters of vaccine have also been found to improve immune responses. In a 1997 study by Bar-Joseph et al., 3 month old Romanov x Awassi cross lambs that received a primary immunization containing a recombinant virus coat protein and then were boosted three weeks later with a partially purified native antigen produced more effective immune responses to antigen. It was found that giving boosters (secondary vaccines) to lambs three weeks after the initial vaccine produced higher titers in sera of Ig compared to lambs that had received single doses of antigen. Lambs that were not primed with a first dose of antigen, but did receive the second booster dose failed to produce a substantial response 12-15 days later.

Like all mammals, the lamb's immune system matures as it ages. Thus, the response to an antigen will vary based on the age of the animal at the time of vaccine administration. J.M. Corpa et al. in 2000 noted differences in the quality of the immune response induced in lambs aged 15 days and 5 months. The older lambs consistently developed a higher and more persistent antibody response to antigen, possibly due to incomplete immune system maturation in the 15 day old lambs. Mutwiri et al. (2000) also found that newborn lambs exhibited systemic immune responses with lower levels of Ig than 5-6 week old lambs.

To account for variation in the maturity of the immune system several studies look at immune profiles as they developed over a period of time. Premier et al. in 2003 designed a study to examine antibody isotype profiles in sheep sera and circulating cells in which sheep were immunized three times at two week intervals with Keyhole Limpet Hemocyanin (KLH), an immunostimulant and carrier protein derived from the mollusk Megathura crenulata and augments both the cellular and humoral components of immune responses (Linn et al., 2000). Premier's study found that the route of vaccine administration affects the immunological response and that antibody secreting cells can be successfully used to assay humoral responses in ruminants.

Using a similar vaccination schedule, Sedgmen et al. in 2005 investigated antibody production in 1-2 year old sheep immunized three times over a seven week period, also with KLH. This schedule allowed the researchers to study antibody response over a period of time to obtain an immune response profile.

Most studies to date have examined antibody production after vaccination. Another approach would be to measure lymphocyte activation. Concanavalin A (con A)

is a commonly used mitogen that is used to measure activation of unprimed T cells in proliferation assays (Kruisbeek et al., 2007). Another mitogen, pokeweed mitogen (PWM) works through T cell dependent activation of B cells (James et al., 2007). Since the activation of lymphocytes occurs before Ig production, examination of proliferative responses of lymphocytes to various mitogens and antigens after vaccination may provide additional information regarding immune competency.

Materials and Methods

Animals

The experiment was a 3 x 3 experiment, with 3 ages at initial vaccination (0 wk, 5 wk, and 10 mo) and 3 vaccination treatments: 1) control (no injection); 2) adjuvant (adjuvant injection); 3) vaccine (adjuvant plus antigen injection). Five sheep were randomly assigned to each of the 6 treatments (Table 1). Both ewe and ram lambs were used, but no attempt was made to stratify genders across treatments. The lambs used as 0 week and 5 week samples were born between October 24 and October 29, 2006. For sampling and processing ease, the week 0 and week 5 age groups were split into separate 0A, 0B, 5A, 5B components. Fifteen 10 month old lambs were also randomly assigned to one of the three vaccine treatment groups (Table I). The sampling dates are outlined in Appendix A. During the study all animals were housed and maintained indoors at Cornell's TNR sheep facility.

Table I: Lamb Assignment to Vaccine Treatment

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Vaccinations and Blood Collection

Sheep in the vaccine and adjuvant treatment groups were immunized subcutaneously in the neck three times at two week intervals with 500μL volumes containing 250μg of KLH (Sigma, MO, USA) with 10% alum hydroxide gel as adjuvant or adjuvant alone.

Blood samples (25mL) were collected using Vacutainer ® tubes; 2 (5ml) red-topped tubes for sera collection and two 10 ml heparin-treated (green-topped) tubes for isolation of lymphocytes. The first two blood samples were obtained immediately prior to the second and third immunizations, respectively and the last, 14 days after the third immunization. . Sera was collected from the clotted blood samples by centrifuging at 1800 xg for 10 minutes and stored at -20°c until analysis. The heparin-treated tubes were processed in the same day to isolate lymphocytes to be used in the proliferation and Ig assays.

Preparation of Lymphocytes

Lymphocyte proliferation in response to KLH was measured using the CellTiter 96 Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI). Lymphocytes were isolated from the non-heparinized blood samples by density gradient centrifugation. First the blood was diluted 1:1 with 10ml RPMI 1640 medium, layered over 10ml of Ficoll- Histopaque and then centrifuging at 2300 xg for 20 minutes. The interphase containing lymphocytes was removed into 10ml RPMI media. The cells were then

pelleted by spinning at 1200 xg for 10 minutes. Red blood cells in the samples were then lysed by the addition of 5ml tris ammonium chloride (TAC), pH 7.0 for 5 minutes at room temperature. Five ml RPMI media was added and the cells were centrifuged at 1200 xg for 10 minutes. The supernatant was removed and then 0.5-1.0ml of a media consisting of RPMI 1640 supplemented with 10% fetal calf serum, 2mM glutamine, 100µg/ml penicillin and 100µg/ml streptomycin was added (RPMI+). The cells were counted using trypan blue exclusion.

Proliferation Assay

Cells at a concentration of 1 x 10e-7 cells/ml were dispensed in 50µl volumes into the wells of a 96-well plate with 50µl of KLH, Concanavalin A (Con A), or poke weed mitogen (PWM). All mitogens were prepared to a concentration of 50µg/ml. Control cultures received media alone. The cell cultures were incubated for 4 days at 37°C under 5% CO_2.

After incubation, the CellTiter 96 Non-Radioactive Cell Proliferation assay was according to the manufacturer's instructions to measure proliferation. Fifteen µl of MTT dye solution was added to each well and the plate was incubated for four hours. The reaction was stopped by the addition of 50µl of the solubilization/ stop solution. The amount of proliferation was determined by quantitation of the absorbance levels at 570nm using a Genios Multi-Detection Microplate Reader (Tecan) and Magellan software.

Immunoglobulin Assay

One million cells (100 µL volume) were dispensed in duplicate to the wells of a 24-well plate. For the blank well, 400µl/well of RPMI+ was added. KLH (50µl) at 50 µg/ml was added to the second well in a total volume of 500µl in each well. The cells were incubated for 7 days and then frozen at -70°C until the Ig content of the supernatant was assayed.

Enzyme-linked immunosorbent assay (ELISA) was used to measure levels of IgG present in the cell culture supernatant. Immulon 1B plates were coated with mouse anti-bovine/ovine IgG (Serotec) at a 1:500 dilution of carbonate buffer and stored overnight at 4°C. The plates were then washed twice with 200µl/well PBS-1% Tween and twice with PBS. Then non-specific binding was blocked by the addition of 50µl/well 1% BSA for 1 hour at room temperature. The plates were then washed and 50µl supernatant diluted 1:1 with PBS-0.1% Tween was added to the plate. The plates were covered with parafilm, and incubated overnight at 4°C.

The following day, the plates were washed again and 50µl/well anti-bovine IgG, A, M conjugated to alkaline phosphatase (Sigma) was added at a dilution of 1:2000 in PBS-0.1% Tween. The plates were covered with parafilm, and incubated overnight at 4°C.

The plates were washed and the amount of bound alkaline phosphatase determined by the addition of p-nitophenyl phosphate substrate. Color development of the substrate was then read at 405nm using Genios Multi-Detection Microplate Reader (Tecan) and Magellan software.

KLH- specific Serum Ab Assay

An ELISA similar to the IgG assay was performed to assess the level of anti-KLH IgG in the serum samples. Immulon 1B plates were coated with 50µl of KLH (Serotec) in a 1:500 dilution of KLH /Carbonate Coating Buffer and incubated overnight at 4°C. The plates were then washed twice with 200µl/well PBS-1% Tween and twice with PBS. Then non-specific binding was blocked by the addition of 50µl/well 1% BSA for 1 hour at room temperature. The plates were then washed and 50µl of serum, diluted 1:10 with PBS-0.1% Tween, was added to the plate. The plates were covered with parafilm, and incubated overnight at 4°C.

The following day, the plates were washed again and coated with 50µl/well anti-bovine IgG, A, M conjugated to alkaline phosphatase (Sigma) at a dilution of 1:5,000 in PBS-0.1% Tween. The plates were covered with parafilm, and incubated overnight at 4°C.

The plates were washed and the amount of bound alkaline phosphatase determined by the addition of *p*-nitophenyl phosphate substrate. Color development of the substrate was then read at 405nm using Genios Multi-Detection Microplate Reader (Tecan) and Magellan software.

Statistical Analysis

Statistical analysis was performed using Minitab v.15 to run an analysis of variance (ANOVA). The data were analyzed within each sampling time (2, 4, 6 wk after the initial vaccination). The statistical model included the effect of age at initial vaccination (0 wk, 5 wk, 10 mo), the vaccine treatment (control, adjuvant, vaccine), and the two-way interaction.

Results

Proliferative Responses of Lymphocytes:

Lymphocytes from lambs vaccinated with KLH starting at age 0 weeks had a higher proliferation index in response to KLH after receiving vaccination booster treatments at 4 and 6 weeks of age (**Fig. 1a**). As well, the proliferation to the mitogen, pokeweed was higher for cells from vaccinated lambs at two weeks of age. At 4 weeks of age, the proliferative response of vaccinated animals to PWM was nearly 2-fold higher than that of lambs receiving adjuvant alone (**Fig. 2a**). Likewise, when lymphocytes from lambs vaccinated starting at 0 weeks of age were exposed to Con A, the proliferation index was nearly two times as high in the vaccine-treated lambs treatment compared to the those receiving adjuvant only at age 4 weeks (**Fig. 3a**).

Lymphocytes from lambs vaccinated with KLH starting at age 5 weeks had a higher proliferation index in response to KLH at 7 and 9 weeks of age (**Fig. 1b**). However, when lymphocytes from these lambs were exposed to PWM, there was no change in the proliferation index regardless of vaccine treatment at all of the three sampling times (**Fig. 2b**). On the other hand, when lymphocytes from these lambs were exposed to Con A, the proliferation index of vaccinated animals was higher than that of lambs receiving adjuvant alone at age 7 weeks (**Fig. 3b**).

Lambs vaccinated with KLH starting at 10 months of age had an almost 3-fold increase in the proliferation index in response to KLH at 46 weeks of age (**Fig. 1c**). When lymphocytes were exposed to PWM, the proliferation index for vaccinated animals was higher for all three sampling points, and notably, was over three times greater than that

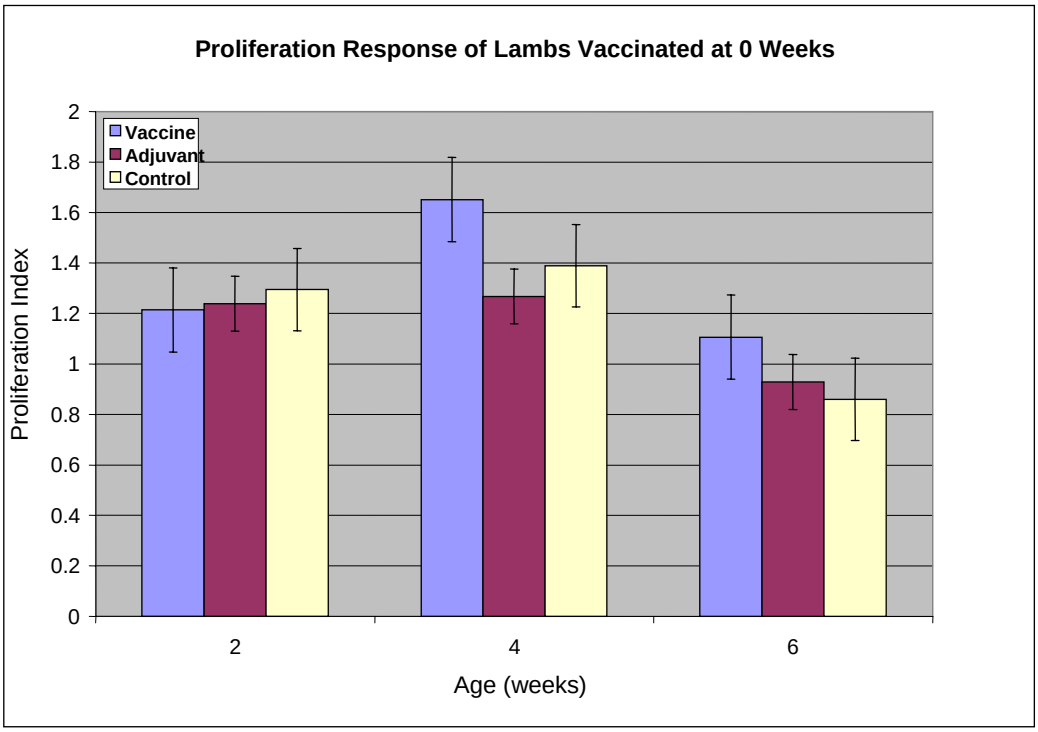
found for lymphocytes from the lambs receiving adjuvant only at the same age (**Fig. 2c**). Likewise, lymphocytes from vaccinated lambs exposed to Con A had a three-fold increase in proliferation index compared to lamb that had received adjuvant only lambs at 46 weeks of age(**Fig. 3c**).

For all three mitogens, the greatest proliferative index was observed in samples taken at age 46 weeks in the animals that had been vaccinated, beginning at 40 weeks (**Fig. 1c, 2c, 3c**).

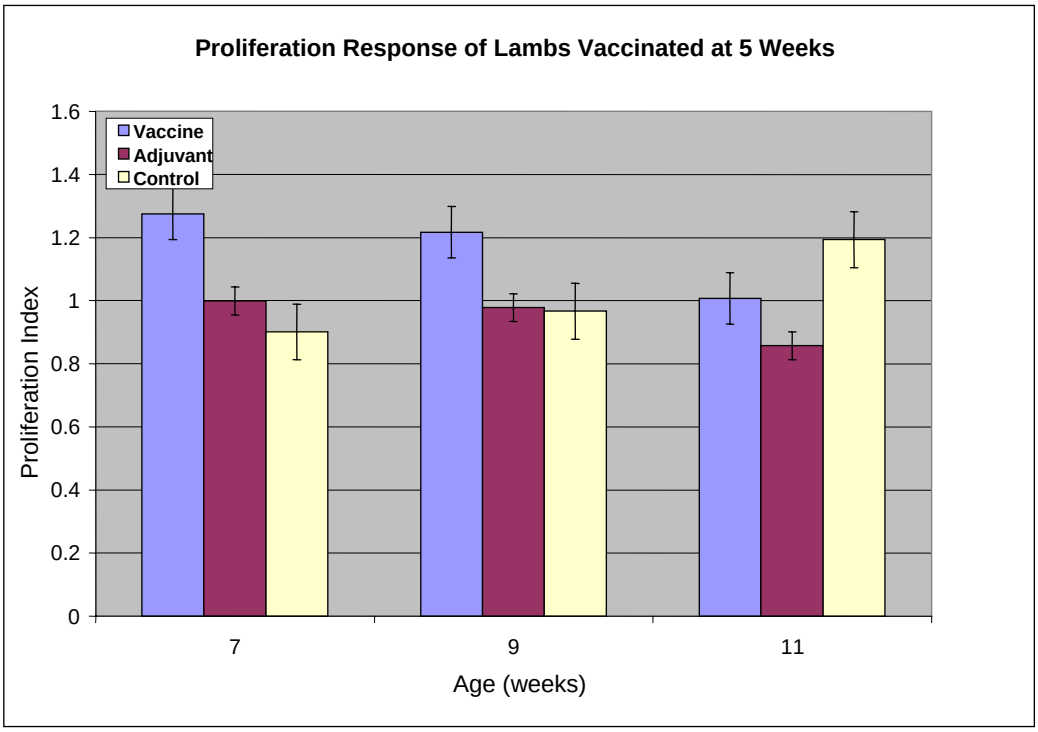
As shown in Table II, ANOVA tests indicate significant ($P < 0.05$) relationships between the age at time of vaccination for the proliferation index of KLH compared to blank.

As well, there was a three-fold increase in the least means squares of lambs vaccinated at 10 months old compared to lambs vaccinated at 0 or 5 weeks of age, regardless of the mitogen examined (**Table III**).

A)


Proliferation Response of Lambs Vaccinated at 0 Weeks
Vaccine
Adjuvant
Control
Proliferation Index
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
4
6
Age (weeks)

B)


Proliferation Response of Lambs Vaccinated at 5 Weeks
Vaccine
Adjuvant
Control
Proliferation Index
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
7
9
11
Age (weeks)

C)

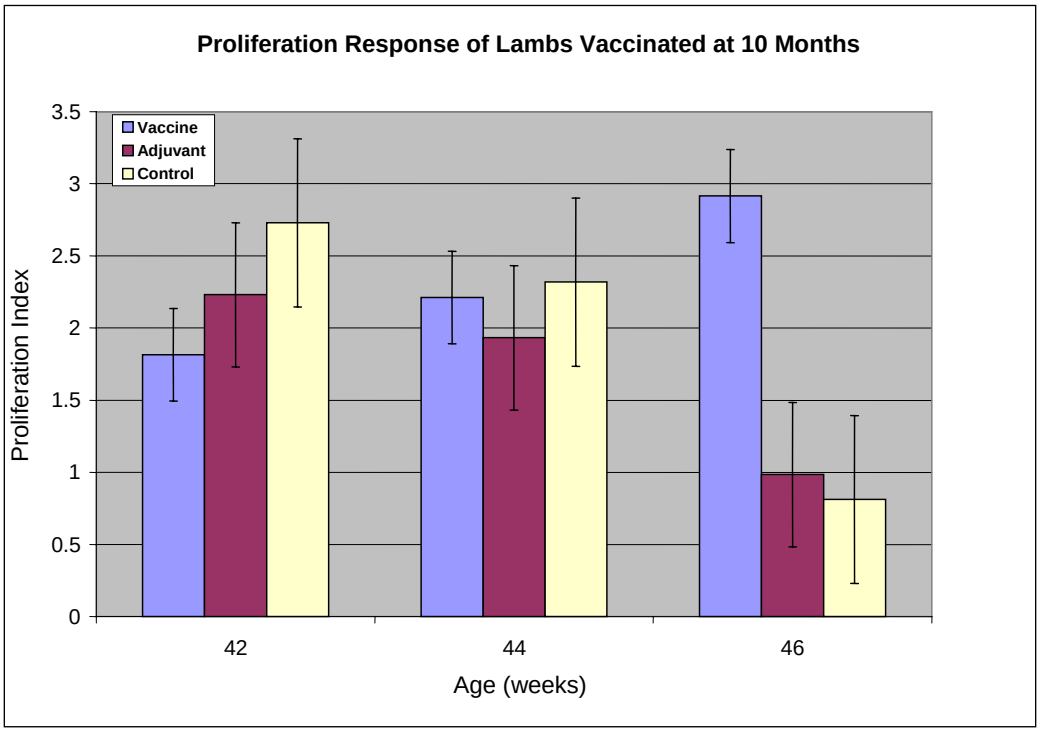
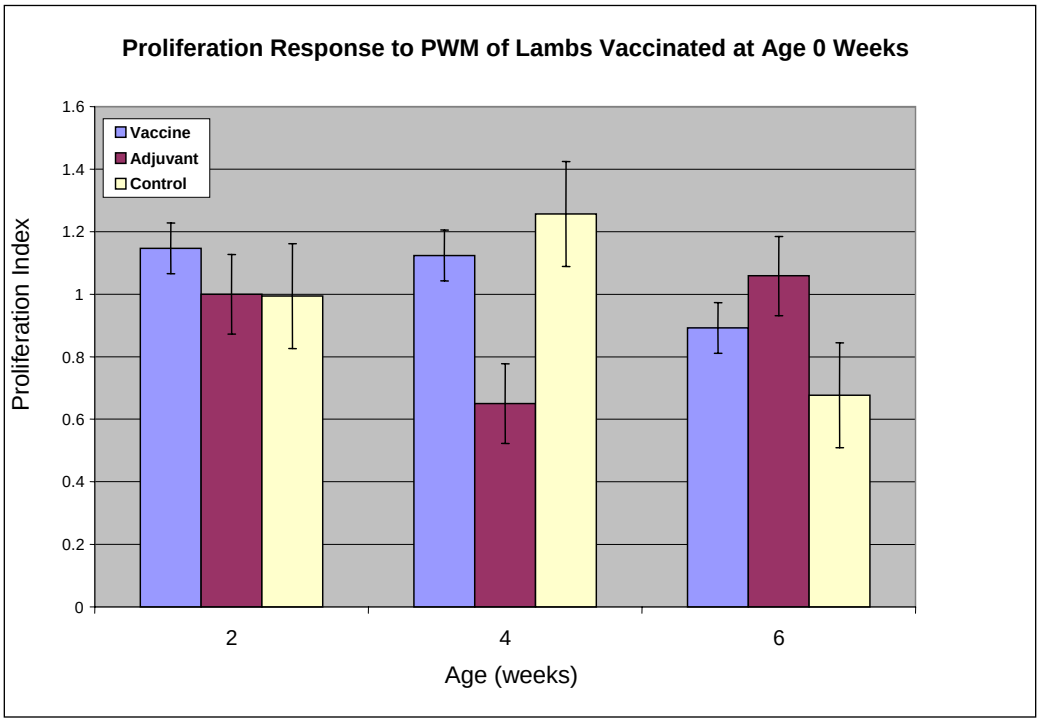
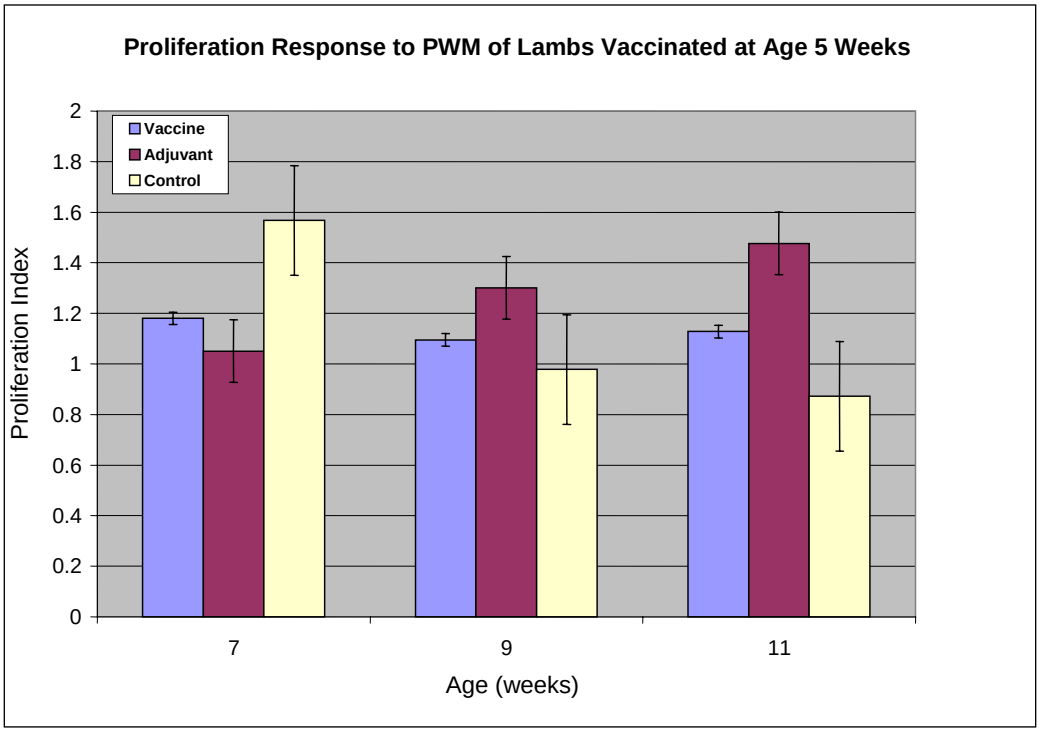


Figure 1: Proliferative response of lymphocytes from lambs vaccinated at A) 0 weeks of age, B) 5 weeks of age, and C) 10 months with KLH in response to KLH. Proliferation was assayed using the Promega 96Cell Titer MTT dye assay and absorbency was read at 570nm. A proliferation index was calculated by comparing KLH proliferation to proliferation under control (blank-no antigen or mitogen) conditions.

A)


Proliferation Response to PWM of Lambs Vaccinated at Age 0 Weeks
Vaccine
Adjuvant
Control
Proliferation Index
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
4
6
Age (weeks)

B)


Proliferation Response to PWM of Lambs Vaccinated at Age 5 Weeks
Vaccine
Adjuvant
Control
Proliferation Index
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
7
9
11
Age (weeks)

C)

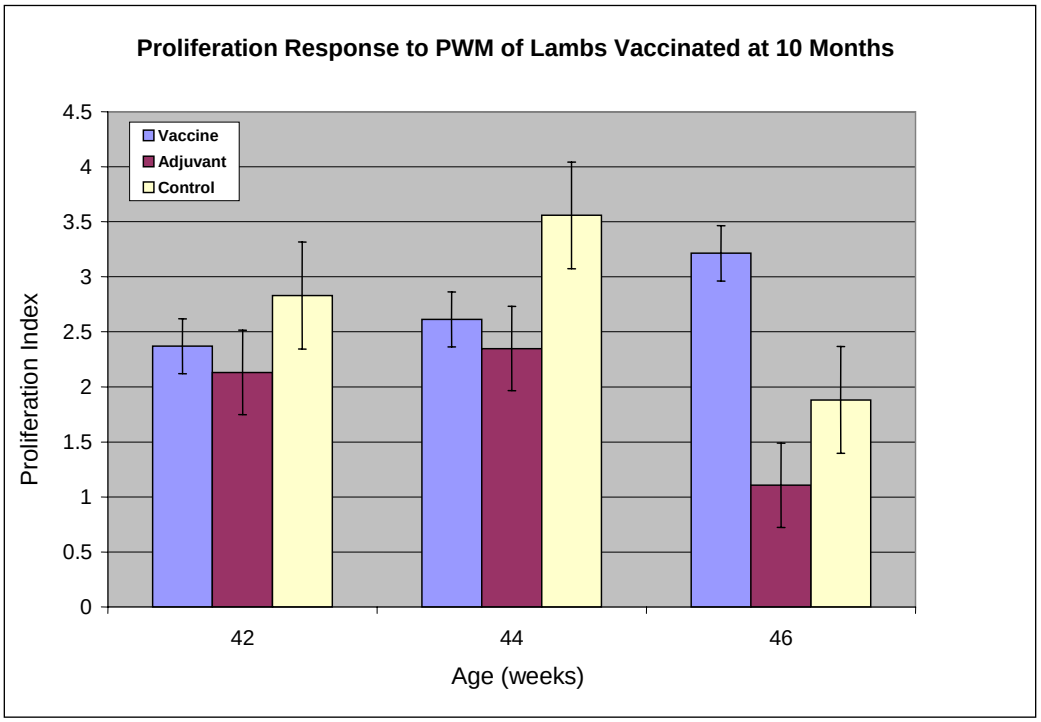
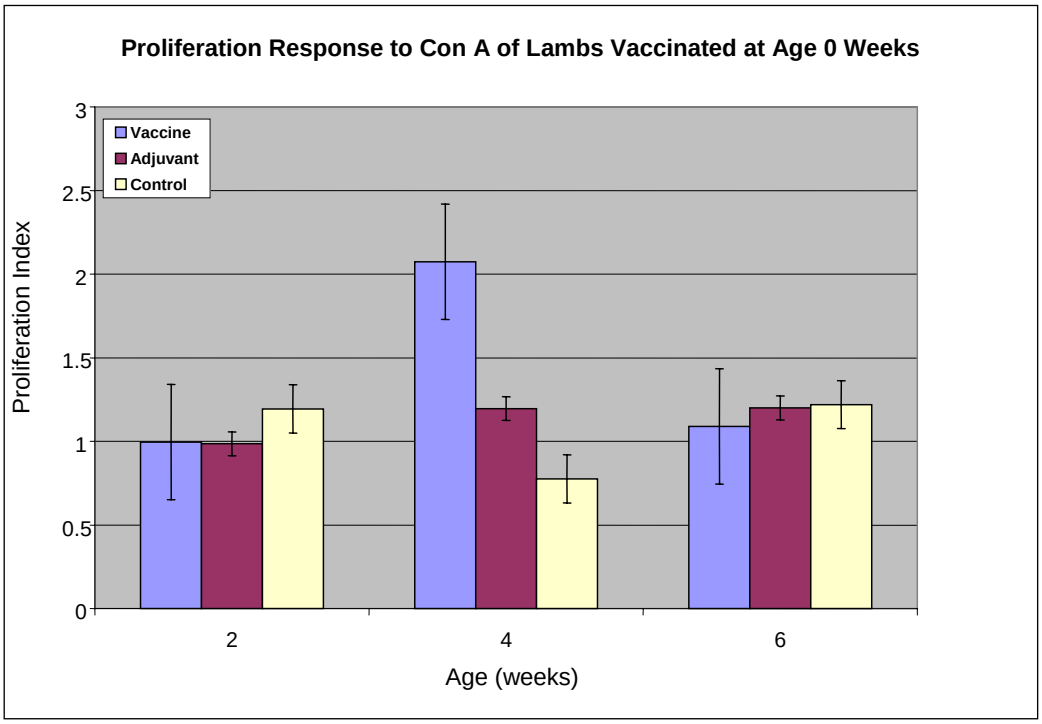
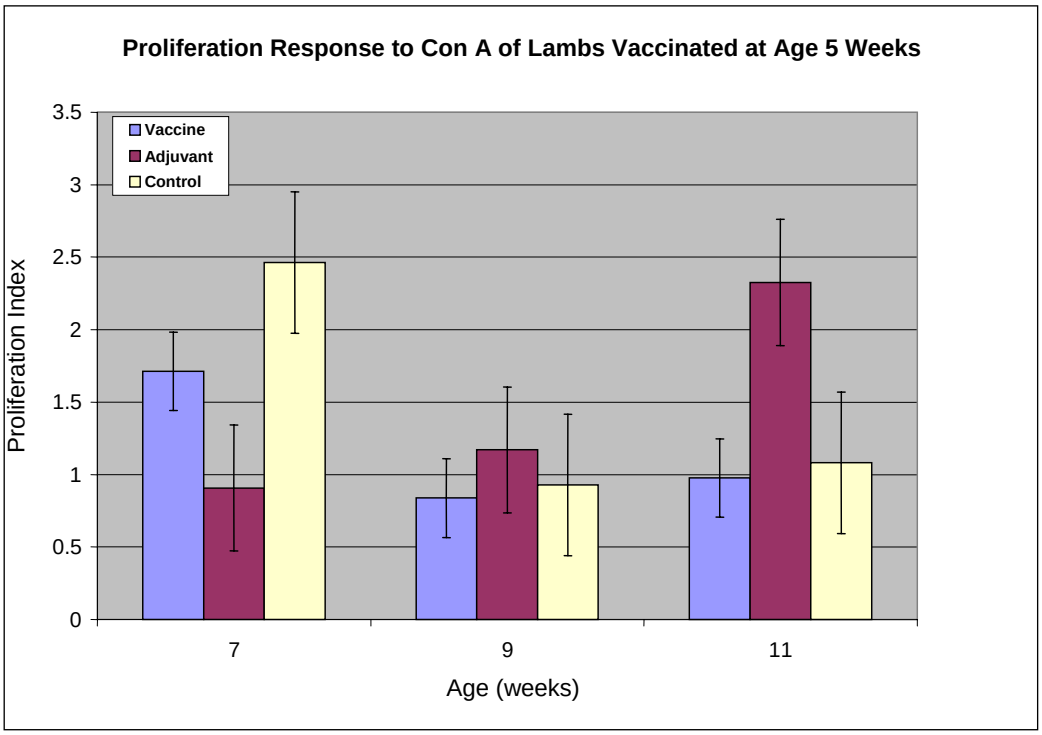


Figure 2: Proliferative response of lymphocytes from lambs vaccinated at A) 0 weeks of age, B) 5 weeks of age, and C) 10 months with KLH in response to PWM. Proliferation was assayed using the Promega 96Cell Titer MTT dye assay and absorbency was read at 570nm. A proliferation index was calculated by comparing KLH proliferation to proliferation under control (blank-no antigen or mitogen) conditions.

A)


Proliferation Response to Con A of Lambs Vaccinated at Age 0 Weeks
Vaccine
Adjuvant
Control
Proliferation Index
3
2.5
2
1.5
1
0.5
0
2
4
6
Age (weeks)

B)


Proliferation Response to Con A of Lambs Vaccinated at Age 5 Weeks
Vaccine
Adjuvant
Control
Proliferation Index
3.5
3
2.5
2
1.5
1
0.5
0
7
9
11
Age (weeks)

C)

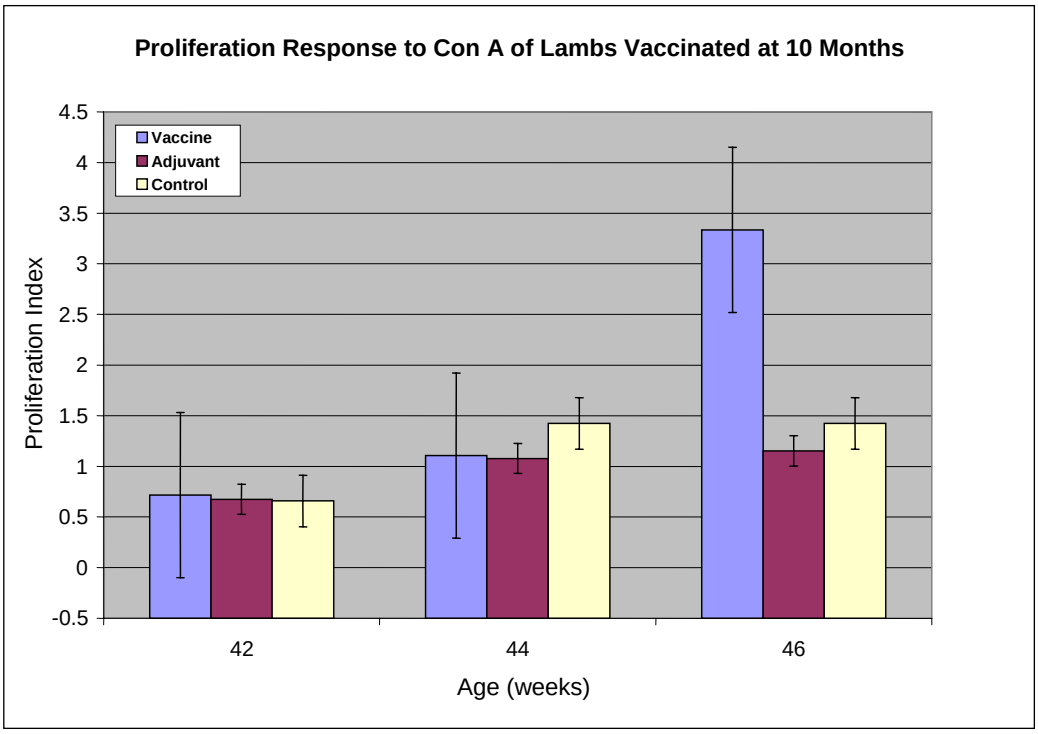


Figure 3: Proliferative response of lymphocytes from lambs vaccinated at A) 0 weeks of age, B) 5 weeks of age, and C) 10 months with KLH in response to Con A. Proliferation was assayed using the Promega 96Cell Titer MTT dye assay and absorbency was read at 570nm. A proliferation index was calculated by comparing KLH proliferation to proliferation under control (blank-no antigen or mitogen) conditions.

Table II: ANOVA for proliferation index of lambs vaccinated with KLH in response to exposure to KLH, Con A, or PWM.

Analysis of Variance for KLH/BLK, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Vac?	2	0.239	0.325	0.163	0.04	0.962
Age	2	28.638	27.518	13.759	3.27	0.05*
Total	4	173.576	143.062	4.208	0.1	0.983
Error	42	143.062	1.638	0.409		
Vac?*Age	34	1.638				
S = 2.05127		R-Sq = 17.58%		R-Sq(adj) = 0.00%		

Analysis of Variance for Con A/BLK, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Vac?	2	4.174	4.753	2.376	0.26	0.771
Age	2	50.156	49.82	24.91	2.74	0.079*
Vac?*Age	4	2.782	2.782	0.695	0.08	0.989
Error	34	308.62	308.62	9.077		
Total	42	365.73				
S = 3.01283		R-Sq = 15.62%		R-Sq(adj) = 0.00%		

Analysis of Variance for PWM/BLK, using Adjusted SS for Tests

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Vac?	2	7.819	8.153	4.077	0.54	0.589
Age	2	33.824	33.112	16.556	2.18	0.128
Vac?*Age	4	8.285	8.285	2.071	0.27	0.893
Error	34	257.73	257.73	7.58		
Total	42	307.66				
S = 2.75325		R-Sq = 16.23%		R-Sq(adj) = 0.00%		

Blood samples were taken two weeks post-vaccination, exposed to mitogen, and proliferative response was assayed using MTT dye.

Table III: Least means squares for proliferation of lymphocytes in response to mitogen exposure

	KLH/BLK		Con A/BLK		PWM/BLK	
Vac?	**Mean**	**SEMean**	**Mean**	**SEMean**	**Mean**	**SEMean**
Adjvnt	1.5529	0.5296	2.469	0.7779	2.2276	0.7109
Control	1.4065	0.5855	1.7341	0.86	1.1287	0.7859
Vaccine	1.6238	0.5296	1.7999	0.7779	1.7447	0.7109
Age						
0	0.9646	0.5296	1.1694	0.7779	0.9776	0.7109
5	0.9667	0.5855	1.3278	0.86	1.2039	0.7859
10	2.6519	0.5296	3.5058	0.7779	2.9195	0.7109

Production of Immunoglobulin (IgG)

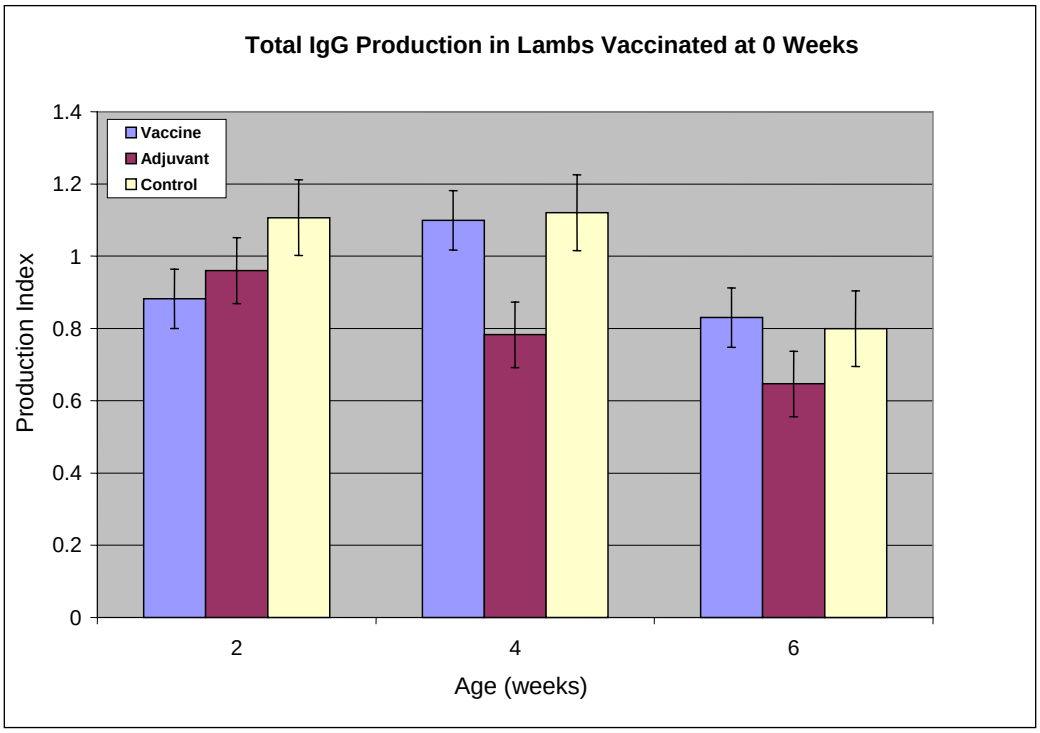
Total IgG production was determined using an ELISA assay. Colorimetric results were read at 405nm and IgG produced in the presence of KLH was compared to control (media alone), and the data reported as fold response IgG over control. Lambs vaccinated with KLH starting at age 0 weeks produced more IgG at age 4 and 6 weeks than lambs receiving adjuvant alone (**Fig. 4a**).

Lambs vaccinated at age 5 weeks tended to produce slightly more (~20%) IgG than lambs receiving adjuvant only at age 11 weeks (**Fig. 4b**).

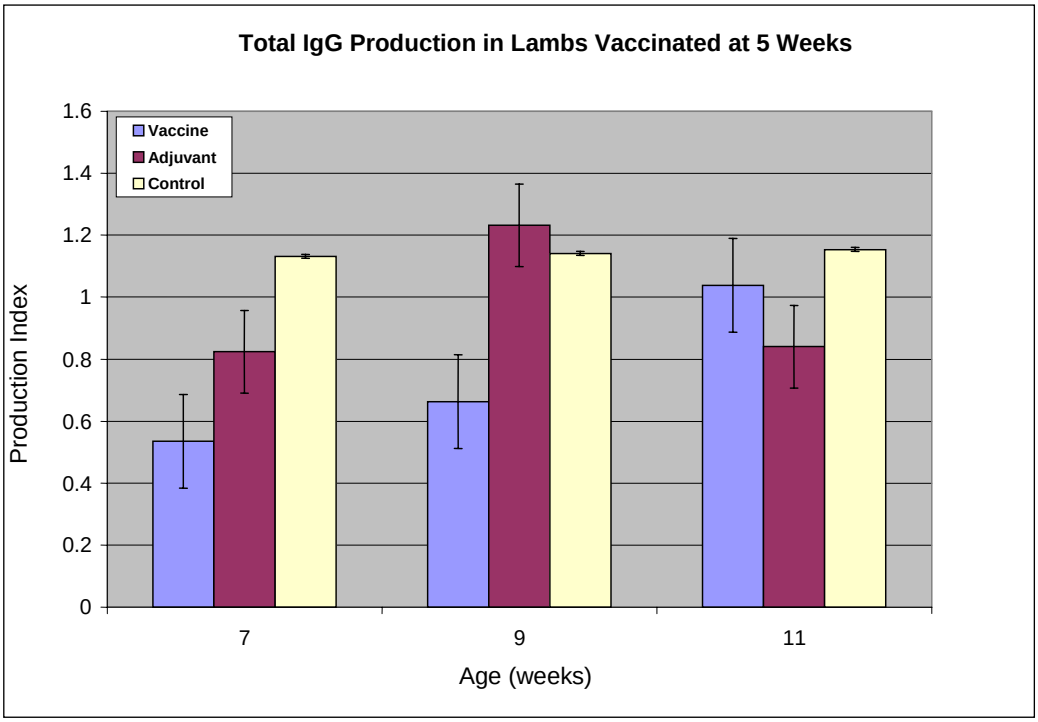
Lambs vaccinated with KLH at 10 months old had greater IgG production than lambs receiving adjuvant only at age 44 weeks (**Fig. 4c**).

Tables IV, V, and VI show that no significant relationships were seen between vaccine treatment, age at time of treatment, nor a cross of the two factors during any sample week during the trials.

A)


Total IgG Production in Lambs Vaccinated at 0 Weeks
Vaccine
Adjuvant
Control
Production Index
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
4
6
Age (weeks)

B)


Total IgG Production in Lambs Vaccinated at 5 Weeks
Vaccine
Adjuvant
Control
Production Index
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
7
9
11
Age (weeks)

C)

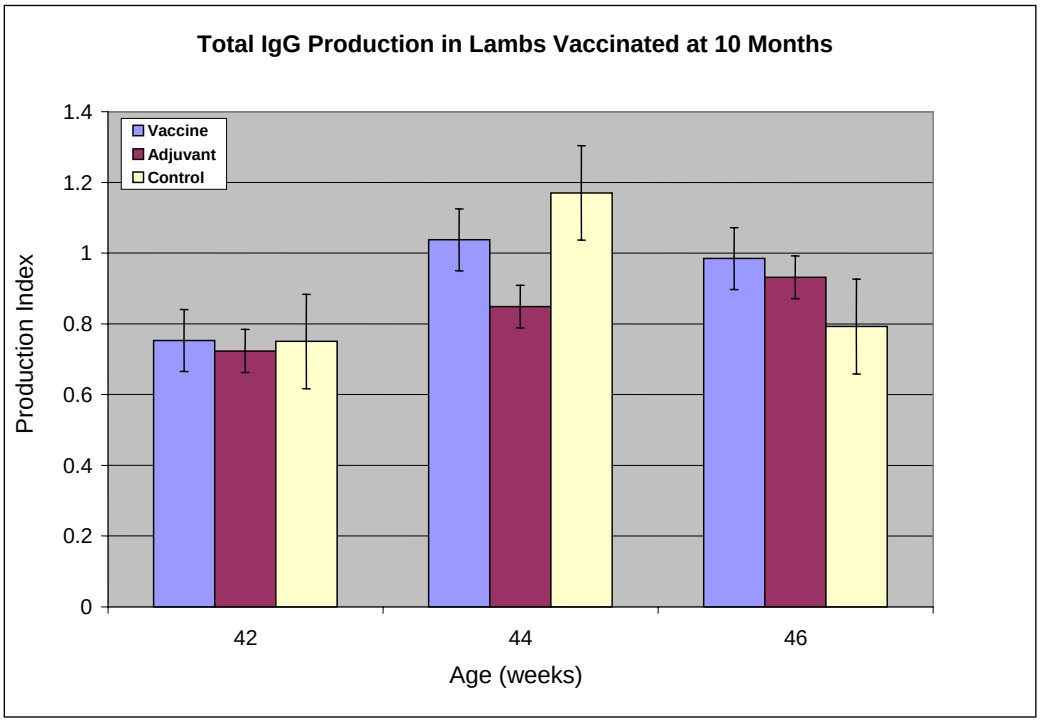


Figure 4: Total IgG production in lambs vaccinated with KLH at A) 0 weeks of age, B) 5 weeks of age, and C) 10 months of age. IgG production was measured by ELISA and expressed as O.D. 405nm; results were expressed as the fold in IgG produced in the presence of KLH over IgG production in control (media alone).

Table IV: ANOVA results for IgG Concentrations Assayed Using ELISA for Samples Collected on Week 2 of Vaccine Trials

Analysis of Variance for KLH/BLK, using Adjusted SS for Tests						
Source	**DF**	**SeqSS**	**AdjSS**	**AdjMS**	**F**	**P**
Vac?	2	0.7037	0.6167	0.3084	0.83	0.446
VacAge	2	0.4071	0.3923	0.1962	0.53	0.595
Vac?*VacAge	4	0.3713	0.3713	0.0928	0.25	0.907
Error	28	10.4004	10.4004	0.3714		
Total	36	11.8826				
S = 0.609462 R-Sq = 12.47% R-Sq(adj) = 0.00%						
Analysis of Variance for KLH-Blank, using Adjusted SS for Tests						
Source	**DF**	**SeqSS**	**AdjSS**	**AdjMS**	**F**	**P**
Vac?	2	0.00416	0.00545	0.00272	0.090	0.916
VacAge	2	0.01804	0.01881	0.0094	0.300	0.74
Vac?*VacAge	4	0.01142	0.01142	0.00285	0.090	0.984
Error	28	0.86581	0.86581	0.03092		
Total	36	0.89943				
S = 0.175846 R-Sq = 3.74% R-Sq(adj) = 0.00%						

Table V: ANOVA results for IgG Concentrations Assayed Using ELISA for Samples Collected on Week 4 of Vaccine Trials

Analysis of Variance for KLH/BLK, using Adjusted SS for Tests						
Source	**DF**	**SeqSS**	**AdjSS**	**AdjMS**	**F**	**P**
Vac?	2	0.8321	0.977	0.4885	2.410	0.107
VacAge	2	0.4464	0.4214	0.2107	1.040	0.366
Vac?*VacAge	4	0.1547	0.1547	0.0387	0.190	0.941
Error	29	5.8665	5.8665	0.2023		
Total	37	7.2997				
S = 0.449769 R-Sq = 19.63% R-Sq(adj) = 0.00%						
Analysis of Variance for KLH-Blank, using Adjusted SS for Tests						
Source	**DF**	**SeqSS**	**AdjSS**	**AdjMS**	**F**	**P**
Vac?	2	0.03361	0.04271	0.02136	1.470	0.246
VacAge	2	0.00624	0.0086	0.0043	0.300	0.746
Vac?*VacAge	4	0.02831	0.02831	0.00708	0.490	0.744
Error	29	0.4206	0.4206	0.0145		
Total	37	0.48875				
S = 0.120430 R-Sq = 13.94% R-Sq(adj) = 0.00%						

Table VI: ANOVA results for IgG Concentrations Assayed Using ELISA for Samples Collected on Week 6 of Vaccine Trials

Analysis of Variance for KLH/BLK, using Adjusted SS for Tests

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Vac?	2	0.244	0.0817	0.0408	0.170	0.842
VacAge	2	1.2738	1.1409	0.5704	2.420	0.102
Vac?*VacAge	4	0.3097	0.3097	0.0774	0.330	0.858
Error	40	9.4481	9.4481	0.2362		
Total	48	11.2756				

S = 0.486008 R-Sq = 16.21% R-Sq(adj) = 0.00%

Analysis of Variance for KLH-Blank, using Adjusted SS for Tests

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Vac?	2	0.002587	0.000997	0.000499	0.090	0.914
VacAge	2	0.025339	0.026606	0.013303	2.410	0.103
Vac?*VacAge	4	0.007414	0.007414	0.001854	0.340	0.852
Error	40	0.220553	0.220553	0.005514		
Total	48	0.255894				

S = 0.0742552 R-Sq = 13.81% R-Sq(adj) = 0.00%

Production of Anti-KLH Ab

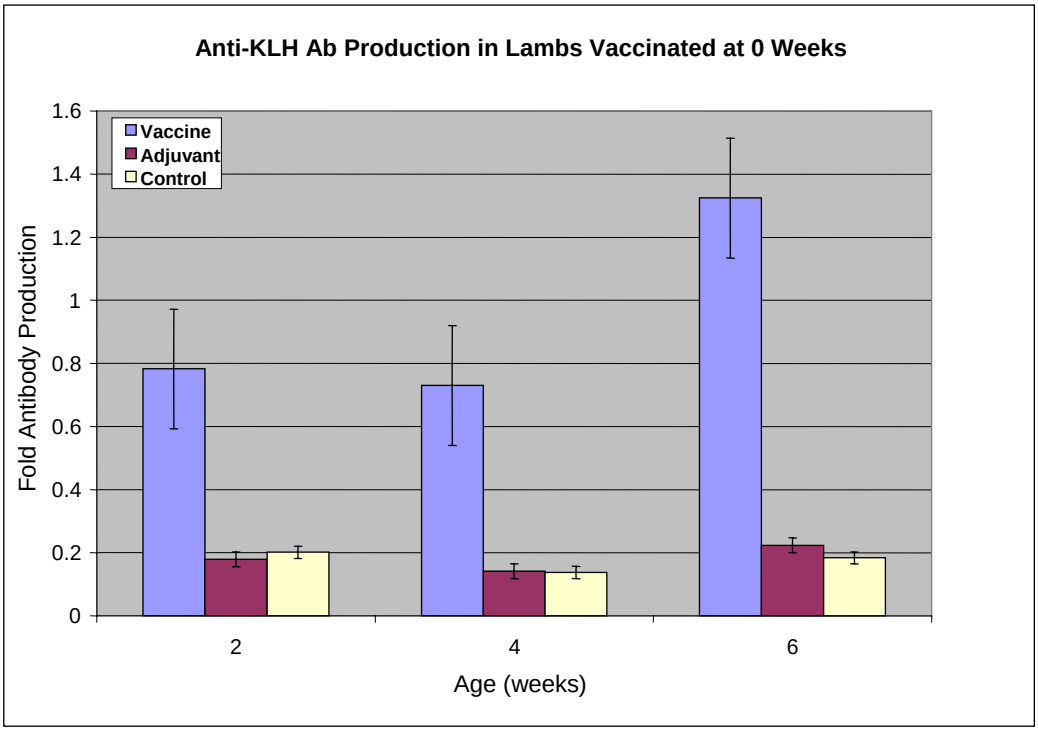
Production of KLH-specific antibodies was measured. ANOVA was conducted to test the effect of vaccine treatment, age at vaccination, and the effect of vaccine treatment and age at vaccination.

Lambs vaccinated with KLH at age 0 weeks did produce anti-KLH Ab in response to the vaccine. At 2 and 4 weeks of age, the response was nearly 4 fold greater that levels produced by lambs that had received adjuvant only. By 6 weeks of age, the response was over 6 times higher in the KLH-vaccinated lambs compared to lambs that had received adjuvant alone (**Fig. 5a**).

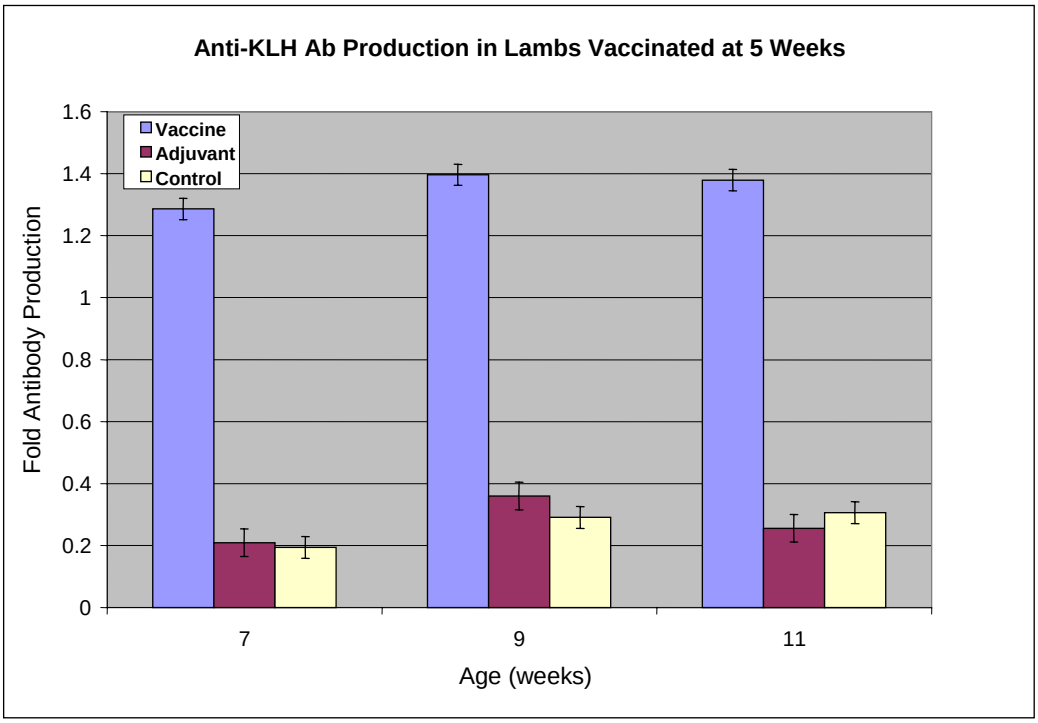
Similarly, lambs vaccinated with KLH at age 5 weeks of age produced nearly seven times as much anti-KLH Ab in response to vaccine than lambs that had received adjuvant alone (**Fig. 5b**). The levels of anti-KLH IgG plateaued so that by week 6 anti-KLH Ab levels were still six to seven times the levels produced in lambs that had received adjuvant alone (**Fig. 5a**). This maximum level of anti-KLH antibody was also seen in the lambs vaccinated at 5 weeks of age, at the 7, 9, 11 week time points, (**Fig. 5b**), and also in the lambs vaccinated at 10 months, at 42, and 44 weeks of age(**Fig. 5c**). Table VII shows that ANOVA shows that there were significant responses to KLH in related to vaccination in lambs vaccinated at all time points; 0, 5 weeks and 10 months of age ($p \leq 0.001$). When age at treatment was examined, there was a significant effect in lambs vaccinated at time 0 ($p \leq 0.001$), while the effect in the lambs vaccinated at 5 weeks of age was almost significant ($p = 0.057$). When these factors were crossed, there was a significant effect in lambs vaccinated at 5 weeks ($p=0.040$) an effect that approached significance for lambs vaccinated at 10 months of age ($p=0.068$).

Figure 3 illustrates the anti-KLH Ab production as the levels of absorbance at 405nm for the ELISA assay.

A)


Anti-KLH Ab Production in Lambs Vaccinated at 0 Weeks
Vaccine
Adjuvant
Control
Fold Antibody Production
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
4
6
Age (weeks)

B)


Anti-KLH Ab Production in Lambs Vaccinated at 5 Weeks
Vaccine
Adjuvant
Control
Fold Antibody Production
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
7
9
11
Age (weeks)

C)

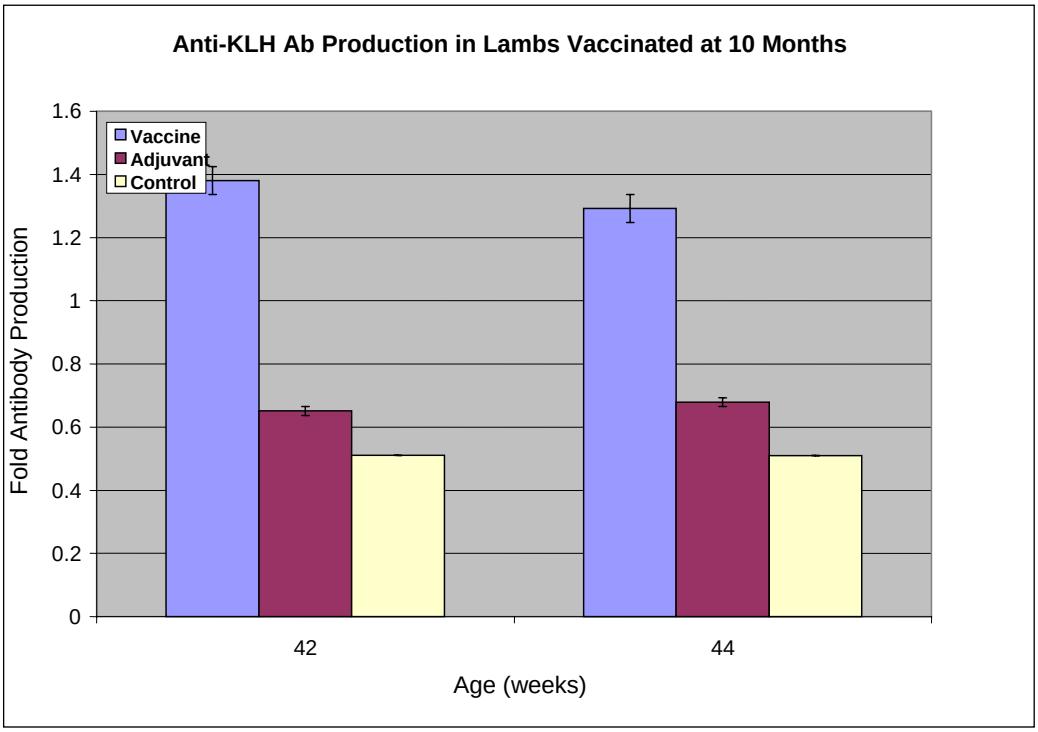


Figure 5: Fold antibody production of anti-KLH Ab in lambs vaccinated with KLH at A) 0 weeks of age, B) 5 weeks of age and, C) 10 months of age. Ab production was determined by ELISA as O.D 405nm; data was expressed as the fold response, anti-KLH IgG produced in the presence of KLH to that produced in control (media alone).

Table VII: ANOVA for anti-KLH AB Production in Sera

Analysis of Variance for KLH, using Adjusted SS for Tests

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Vac?	2	4.79095	4.83025	2.41512	36.86	0.000*
VacAge	2	1.47694	1.47778	0.73889	11.28	0.000*
Vac?*VacAge	4	0.54189	0.54189	0.13547	2.07	0.106*
Error	35	2.29338	2.29338	0.06553		
Total	43	9.10316				

S = 0.255979 R-Sq = 74.81% R-Sq(adj) = 69.05%

Analysis of Variance for KLH, using Adjusted SS for Tests

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Vac?	2	3.8925	3.8762	1.9381	15.860	0.000*
VacAge	2	0.769	0.7584	0.3792	3.100	0.057*
Vac?*VacAge	4	1.3706	1.3706	0.3427	2.800	0.040*
Error	35	4.2761	4.2761	0.1222		
Total	43	10.3083				

S = 0.349536 R-Sq = 58.52% R-Sq(adj) = 49.04%

Analysis of Variance for KLH, using Adjusted SS for Tests

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Vac?	2	6.7725	6.6936	3.3468	52.480	0.000*
VacAge	1	0.0462	0.0378	0.0378	0.590	0.449
Vac?*VacAge	2	0.3865	0.3865	0.1932	3.030	0.068*
Error	23	1.4666	1.4666	0.0638		
Total	28	8.6717				

S = 0.252520 R-Sq = 83.09% R-Sq(adj) = 79.41%

Discussion

Conclusions

The primary objective of this project was to better understand the age at which immunological competency is achieved in postnatal Finnsheep x Dorset lambs. This was examined by creating a vaccine schedule to compare the immune responses of 0 week, 5 week, and 10 month old lambs in response to KLH vaccines.

Vaccinating with KLH- Alum produced an immune response in all three age groups tested. According to the levels of lymphocyte proliferation and anti-KLH Ab in response to a KLH vaccine, lambs receiving vaccines as early as the first week of life can successfully launch an immune response, indicating that vaccines could be administered this early in sheep operations. As mentioned by Chappuis, the antigenicity of vaccines administered early in the lamb's life would have to be increased or paired with a foreign carrier molecule to promote an effective level of protective immunity in newborn lambs.

Additionally, similar to findings by J.M. Corpa, this study found that older lambs (7 weeks- 10 months of age) produced a stronger immune response in terms of higher levels of lymphocyte proliferation, IgG production, and KLH-specific Ab production. Therefore, it may be advantageous to wait approximately two months before administering vaccines that do not encourage as strong of an immune response to ensure that the vaccinations are successful.

Boosting also appears to be a viable method to improve the efficacy of vaccines administered to young lambs. The lymphocyte proliferation index in response to Con A and KLH exposure is greater than that of adjuvant alone by the second sample; four weeks after the first vaccine. This indicates that as Bar-Joseph et al. found, boosting helps

to increase the level of an immune response in lambs. The increased response to Con A indicates increased activation of unprimed T cells. The activated T cells will co-stimulate B cells and lead to a greater immune response. The proliferation index of PWM is greater than that of adjuvant alone starting on the first sample date. This indicates that there is already T cell dependent B cell activation occurring.

Given Fahey and Morris' discovery that lambs can launch antigen-specific immune responses *in utero*, it was expected that the lambs would produce an antibody response to KLH vaccines. As the lambs and their dams were naïve to KLH exposure, no maternally-derived Ab interference was expected. This allowed examination of the lamb's individual immune response and did not reflect the level of passive immunity present.

The anti-KLH Ab data indicates that lambs were producing KLH specific Ab after the first vaccine regardless of age. The response in the 2 week old lambs had half the amplitude of the response in the 6 week old lambs. Once lambs were six weeks or older, the immune response stayed relatively constant around a fold Ab production level of 1.8. This plateau indicates that once lambs reached 6 weeks they were producing the same strength immune response as the mature 10 month old lambs. This is a significant find as it helps determine when lambs exhibit a mature immune profile.

The total IgG data indicates that the adjuvant caused some IgG production. This is unusual as adjuvants are only supposed to serve as immuno-catalysts, increasing the antigenicity of a vaccine but having no response on its' own. Whenever the adjuvant response was higher than the vaccine, the control response was as well. This indicates

that perhaps the total IgG production in lambs ages 7 and 9 weeks was low rather than the adjuvant response being high.

Future Directions for this Research

To further understand the maturation of the lamb immune system, cytokine profiles should also be examined to further indicate when aspects of humoral and cell-mediated immunity are activated by vaccination.

Finally, as this study examined the response to a completely foreign antigen, no maternal Ab blocking effect could be seen. Future research could focus on determining an immune system profile in lambs exposed to an antigen that the dam has been vaccinated against. This would indicate an appropriate timetable for administering the same vaccines that are given to the flock without losing the vaccine's effectiveness to maternal Ab interference.

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APPENDIX:

A: Schedule of lamb vaccination and sampling dates.

Age Group	Vac 1	Smp1/ Vac2	Smp2/ Vac3	Smp 3
Week 0A	30-Oct	13-Nov	27-Nov	11-Dec
Week 0B	9-Nov	22-Nov	8-Dec	20-Dec
Week 5A	5-Dec	19-Dec	2-Jan	16-Jan
Week 5B	14-Dec	29-Dec	11-Jan	25-Jan
Month 10	6-Nov	20-Nov	4-Dec	18-Dec